Characterization of BCAR4, a Novel Oncogene Causing Endocrine Resistance in Human Breast Cancer Cells

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Resistance to the antiestrogen tamoxifen remains a major problem in the management of estrogen receptor-positive breast cancer. Knowledge on the resistance mechanisms is needed to develop more effective therapies. Breast cancer antiestrogen resistance 4 (BCAR4) was identified in a functional screen for genes involved in tamoxifen resistance. BCAR4 is expressed in 27% of primary breast tumors. In patients treated with tamoxifen for metastasized disease high BCAR4 mRNA levels are associated with reduced clinical benefit and progression-free survival. Regarding tumor aggressiveness high BCAR4 mRNA levels are associated with a shorter metastasis free survival and overall survival. In the present study, we investigated the role of BCAR4 in endocrine resistance. Forced expression of BCAR4 in human ZR-75-1 and MCF7 breast cancer cells resulted in cell proliferation in the absence of estrogen and in the presence of various antiestrogens. Inhibition of estrogen receptor 1 (ESR1) expression with small interfering RNA (siRNA) implied that the BCAR4-induced mechanism of resistance is independent of ESR1. Highly conserved BCAR4 homologues of rhesus monkey, green monkey, and the less conserved common marmoset gene induced tamoxifen-resistant cell proliferation, in contrast to the distant BCAR4 homologues of bovine and rabbit. Injection of BCAR4-expressing ZR-75-1 cells into nude mice resulted in rapidly growing tumors. In silico analysis showed that BCAR4 mRNA is highly expressed in human placenta and oocyte, and absent in other normal tissues. In conclusion, BCAR4 is a strong transforming gene causing estrogen-independent growth and antiestrogen resistance, and induces tumor formation in vivo. Due to its restricted expression, BCAR4 may be a good target for treating antiestrogen-resistant breast cancer.


Breast cancer is the second most prevalent cause of cancer death in women in the Western World. Multiple studies demonstrated that the hormone estrogen plays an important role in both breast cancer development and progression. Two estrogen receptor genes, ESR1 (ERα) and ESR2 (ERβ) have been identified, which show similar DNA- and ligand-binding properties, but distinct tissue distributions and functions (Speirs and Walker, 2007). Over two-thirds of invasive breast cancers are ESR1 positive (Harvey et al., 1999). The estrogen dependence of breast cancer represents a unique feature of the disease that can be manipulated by antiestrogens and aromatase inhibitors, to prevent recurrence of the disease. The selective estrogen receptor modulator (SERM) tamoxifen, has been the most commonly used hormonal therapy for ESR1-positive breast cancer during the last three decades (Early Breast Cancer Trialists’ Collaborative Group, 1998; Fisher et al., 1998; Osborne, 1998; IBIS Investigators, 2002; Veronesi et al., 2003; Gradishar, 2005). It competitively binds the ligand-binding domain of the estrogen receptor, which is translocated to the nucleus and interacts with the DNA. This complex binds co-repressor proteins instead of the usual co-activators, resulting in inhibition of transcription and subsequent tumor growth (Arpino et al., 2009).

Although tamoxifen has proven to be effective in the treatment of breast cancer, either intrinsic or acquired resistance to endocrine therapy presents a major challenge in disease management. Several causes of tamoxifen resistance have been identified (Riggins et al., 2007; Musgrove and Sutherland, 2009) including loss of ESR1 expression, ESR1 mutations, alterations in co-regulatory proteins, and increased expression and/or activation of growth factor receptors like EGFR, ERBB2, IGF1R, and FGFR4, and their downstream signaling pathways including ERK and PI3K. The need for therapies circumventing or conversion of the tamoxifen-resistant phenotype remains. Expansion of our insights into the mechanisms underlying therapy failure will help to develop new therapeutic strategies and to overcome endocrine resistance in breast cancer patients.

In order to identify genes responsible for tamoxifen resistance in breast cancer, we performed several functional genetic screens (Dorssers et al., 1993; Dorssers and Veldscholte, 1997; Meijer et al., 2006). Our first screens were based on insertional mutagenesis, an approach in which cells were targeted with defective retroviruses. The rationale for this approach is that virus integration can affect expression of a single gene, consequently altering the cell phenotype (Dorssers et al., 1993; Dorssers and Veldscholte, 1997; Meijer et al., 2006). The second and third screens were performed using a functional gene trap system (IBIS Investigators, 2002) and a screen for genes involved in tamoxifen resistance (IBIS Investigators, 2002).

In the present study, we investigated the role of BCAR4 in endocrine resistance. Forced expression of BCAR4 in human ZR-75-1 and MCF7 breast cancer cells resulted in cell proliferation in the absence of estrogen and in the presence of various antiestrogens. Inhibition of estrogen receptor 1 (ESR1) expression with small interfering RNA (siRNA) implied that the BCAR4-induced mechanism of resistance is independent of ESR1. Highly conserved BCAR4 homologues of rhesus monkey, green monkey, and the less conserved common marmoset gene induced tamoxifen-resistant cell proliferation, in contrast to the distant BCAR4 homologues of bovine and rabbit. Injection of BCAR4-expressing ZR-75-1 cells into nude mice resulted in rapidly growing tumors. In silico analysis showed that BCAR4 mRNA is highly expressed in human placenta and oocyte, and absent in other normal tissues. In conclusion, BCAR4 is a strong transforming gene causing estrogen-independent growth and antiestrogen resistance, and induces tumor formation in vivo. Due to its restricted expression, BCAR4 may be a good target for treating antiestrogen-resistant breast cancer.

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et al., 1993). For these experiments we used human ZR-75-1 breast cancer cells, a cell line fully dependent on estradiol for its proliferation (Van Agthoven et al., 1992). Seven breast cancer antiestrogen resistance (BCAR) genes were identified using this approach (Van Agthoven et al., 1998; 2009b). However, these studies proved to be very laborious in indentifying the genes involved. Therefore, in subsequent screens, we applied a retroviral transduction-based approach utilizing cDNA expression libraries (Brummelkamp and Bernards, 2003). In these screens, cells were transduced with several retroviral cDNA expression libraries. Subsequently, cells were selected for their ability to proliferate in the presence of antiestrogens. When insertion and expression of a cDNA resulted in a proliferating colony of cells, the inserted gene was identified by PCR and nucleotide sequence analysis. Using this approach we previously identified eight additional genes conferring resistance to tamoxifen (Meijer et al., 2006; Van Agthoven et al., 2010). This strategy also led to the identification of the novel gene BCAR4 (Meijer et al., 2006). Antiestrogen-sensitive human ZR-75-1 breast cancer cells transduced with BCAR4 became antiestrogen-resistant and capable of anchorage-independent growth in the absence of estrogen (Meijer et al., 2006). BCAR4 mRNA is expressed in 27% of primary breast tumors. High BCAR4 mRNA levels predict tamoxifen resistance and the presence of BCAR4 mRNA in the primary tumor is associated with tumor aggressiveness (Godinho et al., 2010). In addition, in our cell model BCAR4-induced resistance involves ERBB2/ERBB3 signaling (Godinho et al., 2010).

These observations and the limited knowledge of this novel gene, and the possibility that it may be a target for future therapy prompted us to functionally characterize BCAR4. We analyzed BCAR4 expression in silicone in normal and diseased tissues, and searched for conserved homologues. In addition, the ability of BCAR4 to promote tumor formation in vivo was examined and the possible involvement of ESR1 in the mechanism of resistance to antiestrogens induced by BCAR4 expression was studied.

Materials and Methods

Cell lines and culture conditions

ZR-75-1 cells-derived transfectants containing BCAR4 (ZR/BCAR4) (Meijer et al., 2006), BCAR3 (ZR/BCAR3) (Van Agthoven et al., 1998), or EGFR (ZR/EGFR) (Van Agthoven et al., 1992) were cultured as previously described (Van Agthoven et al., 1992). Human breast cancer cell lines screened for the presence of BCAR4 mRNA (Supporting Table 1) were cultured as described before (Hollestelle et al., 2009).

Generation of BCAR4 constructs and transduction experiments

The coding regions of monkey BCAR4 homologues were retrieved from rhesus monkey and green monkey DNA isolated from blood (obtained from the Biomedical Primate Research Centre, Rijswijk, The Netherlands), and from DNA of the COS-1 cell line (green monkey) using PCR and BCAR4-primate-specific primers (Forward: 5′-TCACCATGTCAACACCTATCC-3′, reverse: 5′-ACAACAGTCTCAGGACATTG-3′, nested forward: 5′-CCATGTACCCAACTGACACCACCC-3′, nested reverse: 1′-CAGACGGAGCTTTCTCAAGAGC-3′, nested reverse: 2′-AGACCAAGATGCGAGGTTC-3′, marmoset forward: 5′-AAGAATCTCAGATCAGACAC3′, marmoset reverse: 5′-AGCTGATGCCGCCGGTC-3′). The rabbit BCAR4 homologue was isolated from DNA from rabbit liver using the following primers: forward: 5′-GGGCGCAGAACGAACCCATGT-3′, reverse: 5′-CACACACCAGCTTTGAGGT-3′. The bovine BCAR4 homologue was a kind gift of Rozenn Dalbies-Tran (Thie et al., 2007). PCR fragments containing the coding sequences were cloned into the pcDNA2.1-TOPO vector (Invitrogen, Breda, The Netherlands), sequenced on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), and from the instructions of the manufacturer and subsequently transferred into the LZRS-RES-Neo expression vector. The expression constructs with rhesus monkey and green monkey BCAR4 were transfected into ZR-75-1 cells, using FuGENE 6 (Roche Diagnostics, Almere, The Netherlands). ZR-75-1 cells containing expression constructs from the common marmoset, rabbit and bovine were generated by viral infection as described before (Meijer et al., 2006; Van Agthoven et al., 2010). After selection for G418 resistance (Invitrogen), colonies were pooled and expanded. Pools of ZR-75-1 cells with BCAR4 or empty vector expression constructs were harvested and plated in 25 cm² tissue culture flasks with medium containing 1 μM 4-hydroxytamoxifen (4-OHT) (Sigma–Aldrich Chemie, Zwijndrecht, The Netherlands) at a density of 1.5 × 10⁵ cells/flask. At days 4, 7, 11, and 14 cells were trypsinized and counted.

Xenograft formation

Cells, resuspended in fresh culture medium and matrigel (5:1) (Collaborative Research, Bedford, MA) were injected subcutaneously (2 × 10⁵ cells/site) under the mammary fat pad of 6-week old female NMR1 nu/nu nude mice (HARLAN-Nederland, Horst, The Netherlands) at the right and left 4th nipple. Developing tumors were measured twice weekly. All animal experiments were performed according to national and institutional regulations.

Quantification of mRNA transcripts

RNA isolation, quantification, cDNA synthesis and normalization to reference genes, were performed as previously described (Sieuwerts et al., 2005). Primer sequences for quantification of ESR1, ESR2, TFF1, and PGR mRNA were published before (Dorsers et al., 2005). For the quantification of BCAR4 transcripts, the Taqman gene expression assay (Hs00415922_m1) from Applied Biosystems was used according to the recommendations of the supplier.

Proliferation assays

ZR/BCAR4 and ZR/vector cells were seeded in 96-well plates at a density of 5,000 cells/well in 200 μl RPMI 1640 (GIBCO, Invitrogen) containing 10% heat-inactivated bovine calf serum (BCS) (Hyclone, Logan, UT) and increasing concentrations of β-estradiol (E2) (from 1 pM to 10 nM), 4-OHT (from 100 pM to 1 nM), 4-hydroxytamoxifen (Sigma–Aldrich Chemie) (from 1 pM to 1 μM) or ICI 182,780 (AstraZeneca, Zoetermeer, The Netherlands) (from 1 pM to 1 μM). One percent ethanol or 0.1% DMSO was used as vehicle controls. To test serum dependence, cells were cultured in 200 μl RPMI 1640 containing increasing concentrations of BCS (from 0.1% to 25%) and 1 nM E2 or 1 μM 4-OHT. For each condition, six replicates were included. After 6 days in culture, a WST-1 proliferation assay (Roche Diagnostics) was performed, according to the instructions of the supplier.

SiRNA-mediated inhibition of cell proliferation

The culture medium of ZR/vector, ZR/BCAR4, and ZR/EGFR cells replaced by medium without E2. After 24 h, cells were seeded into 96-well plates at a density of 7,500 cells/well. The next day, a mixture containing 25 μl of the siRNA dilution, 25 μl of the transfection reagent DharmaFect3 (Dharmacon, Thermo Scientific, Etten-Leur, The Netherlands) dilution, and 50 μl of the supplemented culture medium was added to the wells. Final concentrations were 25 nM for siRNA, 0.1% transfection reagent, 1 nM of E2 or 1 μM 4-OHT. ZR/EGFR cells cultured in 4-OHT-containing medium were supplemented with 10 ng/ml EGF (Roche...
Diagnoses). For each condition, six replicates were included. After 4 days, a WST-1 proliferation assay was performed. SiESR1 (s4825) was purchased from Applied Biosystems International. SiBCAR3 On TARGET plus SMART pools (L-011469-00) was from Dharmacon. To monitor gene silencing, eight wells were pooled by lysis with RNABee (Bio Connect, Huisken, The Netherlands) 48 h after transfection and RNA was isolated according the protocol of the supplier.

Results

Placental and oocyte-specific expression of BCAR4

BCAR4 was identified using functional genetic screens to isolate genes capable of inducing antiestrogen resistance in human breast cancer cells. Retroviral CDNA expression libraries from human brain, placenta, the cervical cancer cell line HeLa, and mouse embryo were used. BCAR4 was isolated from the human placenta library only (Meijer et al., 2006), suggesting tissue and/or species-specific expression. In order to analyze the BCAR4 mRNA expression in normal and diseased tissues, we searched for expression data in the literature, as well as in public expression databases (LOC400500/BCAR4/Hs.24611 and probe set 230854 at on Affymetrix U133 2.0+ platform in Gene Expression Omnibus, GEO, NCBI. NextBio; www.nextbio.com, and SAGE, www.sagenet.org). In normal tissues, BCAR4 was only found to be highly expressed in the oocyte (GEO, series GSE11450) (Kocabas et al., 2006; Zhang et al., 2007) and in the basal plate of the placenta from midgestation to term (GDS2529) (Miner and Rajkovic, 2003; Nishizawa et al., 2007; Milheev et al., 2008). In the expression datasets GSE7307 and 3526, which included 677 tissue samples of 90 normal and diseased human tissues, BCAR4 expression was detected only in placenta and in the MDA-MB-231 breast cancer cell line.

To establish if BCAR4 is also expressed in human breast cancer cell lines, we tested a panel of 42 cell lines. Five (T47D, MDA-MB-415, UACC812, BT474, and ZR-75-30) were found to be positive. These BCAR4 homologues, InterProScan (Quevillon, 2005, http://www.ebi.ac.uk/Tools/InterProScan/) predicted two transmembrane domains and a signal peptide (Fig. 1). The BCAR4 gene is located on human chromosome 16p 13.13, between the genes zinc finger CCH นาย-containing 7A (ZC3H7A) and ribosomal L1 domain-containing protein 1 (RSL1D1). RSL1D1 is oriented head to tail, 23 kb from the 5′-end of BCAR4 and ZC3H7A is oriented head to tail, at the 3′-end, 46 kb of BCAR4. In addition, two related sequences, without an open reading frame, are located on chromosomes 5p12 and 14q23.1. The BCAR4 homologues of chimpanzee, orangutan, rhesus monkey, bovine, horse, American pika and rabbit are also located between the two well conserved genes ZC3H7A and RSL1D1. For the tree shrew and Hoffmann’s two-toed sloth, the gene maps are not yet available, therefore care has to be taken when interpreting that these genes are the actual BCAR4 homologues. BLAST searches failed to identify conserved BCAR4 homologues in the mouse and rat. Although the BCAR4 flanking genes ZC3H7A and RSL1D1 are also well conserved in these species, no BCAR4 homologue could be identified in this locus.

Using PCR, we retrieved the BCAR4 coding sequences from rhesus macaque and green monkey, and generated expression constructs. Estrogen-dependent, antiestrogen sensitive human ZR-75-1 breast cancer cells were transduced with these different expression constructs. Proliferation assays showed that ZR-75-1 cells containing BCAR4 homologues derived from rhesus macaque and green monkey were able to proliferate in the presence of 4-hydroxytamoxifen (4-OHT), in contrast to empty vector containing cells (Fig. 2A). These primate homologues retained the capability of inducing tamoxifen resistance; therefore we next tested a less conserved BCAR4 homologue from the common marmoset and distant homologues from rabbit and bovine. Despite 25% sequence divergence, forced expression of the common marmoset BCAR4 still induced tamoxifen resistance (Fig. 2B). However, forced expression of the distant BCAR4 homologues of bovine and rabbit did not confer tamoxifen resistance (not shown), indicating possible divergence of function of BCAR4 or its target(s).

BCAR4 promotes xenograft tumor growth in nude mice

Previously we reported that BCAR4-overexpressing cells are anchorage-independent and form colonies in soft agar (Meijer et al., 2006). We next investigated whether ZR/BCAR4 cells could induce xenograft formation in partially immune-deficient nude mice. Injection of parental ZR-75-1 cells in nude mice does not result in tumor formation. Only when an estrogen-release pellet is administered tumors will develop (not shown). ZR/BCAR4 cells, containing the human BCAR4 CDNA, or empty expression vector containing cells (ZR/vector) were injected into two mammary fat pads of female mice. Within 2 weeks, four out of five mice injected with ZR/BCAR4 cells developed tumors. Three on both sides and one mouse showed tumor formation on one side (Fig. 3A). In one mouse no tumors were detected within a period of 6 months. Mice injected with ZR/vector cells did not develop tumors over a period of 6 months follow-up (Fig. 3B). In a second experiment using seven mice the number of injected BCAR4 cells was increased from 2 to 5 x 10^5 and a take rate of 100% was achieved (not shown). These results show that BCAR4 induces tumor growth in vivo, independently of additionally administered estrogen.
BCAR4 induces estrogen-independent, antiestrogen-resistant proliferation

Previously we showed, that ectopic expression of BCAR4 enables ZR-75-1 cells to proliferate in the presence of 4-OHT (Meijer et al., 2006). To solidify this initial observation and further establish the role of BCAR4 in antiestrogen resistance, we used estrogen-sensitive MCF7 cells. We generated MCF7/BCAR4 cells by transfection and tested these, together with ZR/BCAR4 cells, for proliferation in the presence of the antiestrogen ICI182,780, which is a better growth inhibitor for MCF7 cells than 4-OHT. As shown in Supporting Figure 2A, ZR/BCAR4 cells were fully resistant to this pure antiestrogen. Similarly, MCF7/BCAR4 cells were able to proliferate in the presence of ICI182,780 while vector-control cells were growth inhibited (Supporting Fig. 2B). From these results it is shown

**Fig. 1.** Alignment of predicted protein sequences of different primate BCAR4 homologues. Predicted protein sequences from human (H.s.), chimpanzee (P.t.), gorilla (G.g.g.), Sumatran orangutan (P.p.a.), olive baboon (P.a.), rhesus macaque (M.m.), crab-eating macaque (M.f.), green monkey (C.e.), and common marmoset (C.j.) are shown. Sequences were aligned with the ClustalW2 multiple sequence alignment software tool. Amino acid identities are shown as dots (•) Residues identical in all sequences in the alignment, (+) conserved substitutions, (±) semi-conserved substitutions. The position of the signal peptide, predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), is shown above the sequence (amino acids 1–41, solid line). The transmembrane domains, as predicted by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), are indicated with a dashed line (amino acids 27–46 and 59–77).

**Fig. 2.** Expression of primate BCAR4 homologues induce tamoxifen resistance in human ZR-75-1 breast cancer cells. A: The proliferation curves of ZR-75-1 cells expressing the rhesus macaque (rm) or green monkey (gm) BCAR4 homologues show that these cells are able to proliferate in the presence of 4-OHT, while control cells (ZR/vector) are not. B: ZR-75-1 cells expressing the BCAR4 homologue from common marmoset (cm) can also proliferate in the presence of 4-OHT, in contrast to control cells. For each primate BCAR4 homologue, two independent cell lines were tested. All cell lines were cultured in 4-OHT-containing medium, and counted at the indicated days. Average of three replicates and SDs are presented. We performed an analysis of variance of the data of day 14, which was significant for rm and gm compared to the control (P < 0.0000) and cm (P < 0.0004). Cell doubling times were calculated with exponential regression analysis: rm 85 and 87 h, gm 81 and 83 h and vector control 125 h.
that ectopic expression of BCAR4 transforms both breast cancer models from an antiestrogen-sensitive to an antiestrogen-resistant state.

To further explore the effects of BCAR4 expression, we performed dose–response assays and measured the effects of estrogen, the antiestrogens 4-hydroxytamoxifen, the active metabolite of tamoxifen, raloxifene, and the pure antiestrogen ICI182, 780, and bovine calf serum (BCS). As shown in Figure 4A, addition of E2 to the culture medium, at concentrations up to 1 nM, caused only a minor increase in proliferation rate of ZR/BCAR4 cells, showing their estrogen-independent phenotype. In contrast, addition of 1 nM of E2 to the culture medium of empty vector-containing ZR-75-1 cells strongly enhanced their proliferation rate. ZR/BCAR4 cells were not inhibited by the addition of the antiestrogens 4-OHT (Fig. 4B) and raloxifene (Fig. 4C). Addition of the pure antiestrogen ICI182, 780 up to 1 μM slightly stimulated proliferation of ZR/BCAR4 cells (Fig. 4D). ZR/vector cells were unable to proliferate in the presence of antiestrogens (Fig. 4B–D). Similar to the parental cells, the proliferation capacity of ZR/BCAR4 cells was fully dependent on the presence of BCS in the culture medium (Fig. 4E). BCS concentrations up to 6.25% resulted in a clear increase of both ZR/BCAR4 and parental cell proliferation, but higher concentrations had no further effect. In E2-containing medium, ZR/BCAR4 cells have an increased proliferative capacity compared with ZR/vector cells (Fig. 4E). This result shows that BCAR4 expression confers cells a proliferative advantage. In 4-OHT-containing medium, ZR/BCAR4 cells proliferate at the same rate as control cells when cultured in estrogen-containing medium. Increasing concentrations of BCS were unable to overcome the inhibition of proliferation caused by 4-OHT of empty vector containing cells.

**ESR1** is functional in cells with forced expression of BCAR4

In general, clinical tamoxifen resistance is not due to loss of ESR1 expression (Robertson, 1996; Johnston et al., 1997). In estrogen receptor-positive breast cancer cell lines the expression of progesterone receptor (PGR) and trefoil factor 1 (TFF1), former name PS2 is induced by estrogen. In primary breast tumors expression of ESR1, PGR, and TFF1 predicts the presence of a functional ESR1 signaling pathway (Horwitz and McGuire, 1978; Masiakowski et al., 1982; Foekens et al., 1990, 1993; Van Agthoven et al., 1994; Miller et al., 2007; Walker et al., 2007).

In order to investigate if the receptor is still present and functional in ZR-75-1 cells expressing BCAR4, we measured the mRNA expression levels of ESR1, ESR2, and the estrogen-regulated genes PGR and TFF1. In all the cell lines tested, ESR1 expression was present (Fig. 5A). In ZR/BCAR4 cells ESR1 expression was slightly lower than in ZR/vector control cells. The addition of 4-OHT to the culture medium resulted in decreased ESR1 expression levels in all cell lines, compared with E2-containing cultures. ESR2 expression was not detected (not shown). In all cell lines, the expression of TFF1 (Fig. 5B) and PGR (Fig. 5C) was higher in the estrogen-treated cultures than in the 4-OHT-treated cultures. These results indicate that the antiestrogen resistance observed in the ZR/BCAR4 cells is not accompanied by loss of ESR1 expression or loss of function.

**ESR1 silencing does not affect the proliferation rate of BCAR4 overexpressing cells**

To establish whether BCAR4 expression activates ESR1 in a non-estrogen-dependent manner, we inhibited ESR1 expression with specific siRNAs. Using RT-PCR, we determined that the inhibition of ESR1 was >90% in ZR/BCAR4 and ZR/vector cells, and 70% in ZR/EGFR cells. In E2-containing medium, the proliferation capacity of ZR/BCAR4 cells was not affected by the inhibition of ESR1. In contrast, siESR1 significantly inhibited proliferation of empty vector-containing cells and EGFR expressing cells (Fig. 6A). In the presence of 4-OHT the proliferation of BCAR4 expressing cells was also not inhibited by siESR1 (Fig. 6B). Inhibition of ESR1 in the presence of 4-OHT and EGF did not reduce proliferation of ZR/EGFR cells (Fig. 6B), indicating that the stimulation with EGF allowed cells to bypass the dependence on estrogen receptor signaling. ZR/vector cells are fully inhibited by antiestrogens, and in 4-OHT-containing medium the inhibition of ESR1 had no additional effect on cell proliferation (Fig. 6B). Control transfections with siBCAR3 did not change the growth properties of these cell lines, indicating absence of non-specific effects. Our results indicate that in our model, the mechanism of resistance to tamoxifen induced by BCAR4 and EGFR expression is independent of the ESR1 signaling.

**Discussion**

In the present study, we aimed to functionally characterize the novel gene BCAR4, in order to elucidate its role in endocrine...
resistance and normal development. The analysis of public databases and the cloning and sequencing of BCAR4 homologues confirmed the presence of this gene in several mammalian species. While highly conserved in higher primates, the protein sequence of the New World monkey common marmoset already showed substitution of 25% of the amino acids. Due to the occurrence of substitutions, deletions and insertions, protein sequences of other non-primate mammalian species were very difficult to align with the human sequence. Strikingly, the BCAR4 gene was lost in mouse and rat possibly due to a deletion event, while the flanking genes (ZC3H7A and RSL1D1) were retained. BCAR4 has a predicted coding region of 121 amino acids, resulting in a small protein of 13 kDa. The analysis of the predicted amino acid sequence of the BCAR4 proteins do not reveal conserved domains, with the exception of two transmembrane domains and a signal peptide, indicating that the protein may be localized at cell membranes. Our in vitro assays confirmed that all the primate homologues tested were able to

Fig. 4. Effects of estrogen, antiestrogens and serum on the proliferation of ZR/BCAR4 cells. Closed lines represent ZR/vector cells and dashed lines ZR/BCAR4 cells. In all experiments cell viability was determined with the WST-1 proliferation assay on day 6. A: ZR/BCAR4-expressing cells are independent of estrogens, in contrast to empty vector containing control cells. B: No dose-dependent effects on ZR/BCAR4 cell proliferation were observed upon addition of increasing concentrations of the antiestrogens 4-OHT or (C) with raloxifene. D: A minor stimulation of BCAR4 cells was observed in the presence of ICI182, 780. E: ZR/vector control cells and ZR/BCAR4 cells were cultured with estrogen (1 ng/ml) or 4-OHT (1 μM)-containing medium, and in the presence of increasing concentrations of bovine calf serum. The main finding of the last experiment is that ZR/BCAR4 cells are still dependent on serum components for proliferation.
BCAR4 was the most abundantly recovered gene from our functional screen with the placenta cDNA library (Meijer et al., 2006; Van Agthoven et al., 2010), suggesting tissue-restricted expression. Microarray data also showed high expression in placenta and absence of expression in most other tissues. Further studies with specific antibodies directed against BCAR4 are needed to confirm these mRNA expression data. High expression of BCAR4 in several stadia of the placenta (GDS2529) suggests an important role for this gene in this tissue. Therefore the absence of BCAR4 in rodents is remarkable. Differences in placental development, morphology and function may explain the absence of the gene in rodents. A small series of samples showed that low BCAR4 mRNA levels are associated with preeclampsia (GDS2080) (Nishizawa et al., 2007), a common severe complication of pregnancy which originates in the placenta (Young et al., 2010). In addition to placenta, oocytes of both human and bovine were found to express high levels of BCAR4 (GSE1450, GSE18290) (Kocabas et al., 2006; Thelie et al., 2007; Zhang et al., 2007). A time-course human expression dataset of the one, two, four, and eight-cell stage, morula and blastocyst showed that the level of mRNA of BCAR4 declines rapidly during the morula stage and is absent in the blastocyst (GSE18290). The presence of a stable mRNA pool in the mature oocyte to provide the fertilized egg with newly synthesized proteins is well established (Braude et al., 1988; Gandolfi and Gandolfi, 2001; Schier, 2007). The maternal pool of RNAs and proteins supports embryonic growth from fertilization until the moment that transcriptional activity of the embryonic genome is activated (Gandolfi and Gandolfi, 2001; Schier, 2007). BCAR4 is one of the seventy highest mRNAs present in the human oocyte (Brinkman et al., 2010). Therefore, BCAR4 may have an important role in early development. In humans, degradation of maternal RNAs and activation of embryonic genome activity occurs around the four- and eight-cell stage. The degradation of maternal RNAs removes gene products that may interfere with processes occurring at this developmental stage, or because its RNA is expressed in a minority of cells, resulting in undetectable levels in total cell lysates.

Besides its expression pattern in normal development, we aimed to unravel the function of BCAR4 in antiestrogen resistance in human breast cancer and thereby find leads for novel treatment possibilities. BCAR4 expression transformed estrogen-dependent human ZR-75-1 and MCF7 breast cancer cells into an estrogen-independent phenotype. The involvement of ESR1 in the resistance to tamoxifen, due to its cross talk with growth factor signaling was documented (Massarweh and Schiff, 2006). In our model, the inhibition of ESR1 with specific siRNAs had no effect on BCAR4-induced cell proliferation, showing that this mechanism is independent of the ESR1. In addition, ZR/BCAR4 cells were not only resistant to tamoxifen (Meijer et al., 2006), but also to raloxifene and to the selective estrogen receptor down-regulator ICI182.780 (fulvestrant/Faslodex). ICI182.780 targets the estrogen receptor by a different mechanism than other SERMs. It inhibits receptor dimerization and abrogates estrogen signaling (Kansra et al., 2005), further supporting an ESR1 independent mechanism.

Loss of ESR1 expression has also been indicated as one of the mechanisms of acquired tamoxifen resistance (Musgrove and Sutherland, 2009). The in vitro data presented, showed that antiestrogen resistance induced by BCAR4 is independent of and induce antiestrogen-resistant cell proliferation. However, forced expression of BCAR4 homologues of rabbit and cow failed to accomplish this, indicating that preservation of the putative transmembrane and signal peptide domains is not sufficient to induce tamoxifen resistance in human ZR-75-1 cells.

**Fig. 5.** ESR1 is present and functional in ZR/BCAR4 cells. A: ESR1 was expressed in all cell lines tested. B: Expression of the estrogen-regulated genes TFF1. C: PGR was enhanced in estrogen-treated cells (open bars) compared to cells in the presence of 4-OHT (closed bars). Average of four replicates and standard deviations are presented.
progression of the disease. Three out of the five panel, suggesting a role for this gene in the development or (Godinho et al., 2010) and in 12% of our breast cancer cell line ESR1-positive and ESR1-negative primary breast cancers (Slamon et al., 1989; Gullick et al., 1991; Berns et al., 1992, 1995; Dowsett et al., 2001; Hurtado et al., 1992; Berns et al., 1995; Liu et al., 2007; Van Agthoven et al., 2009a). Gene amplification and over-expression of ERBB2 has been shown to contribute to a poor clinical outcome (Klijn et al., 1995), this novel gene may have clinical value in breast cancer sarwari and Charlotte van Putten is greatly appreciated. We thank Dr. Antoinette Hollestelle and Dr. Mieke Schutte for use of the breast cancer cell line panel. We also thank Prof. Dr. Leendert Looijenga for helpful comments regarding the manuscript.

Our previous data showed that forced expression of BCAR4 resulted in strong phosphorylation of ERBB2, ERBB3, and the downstream mediators of ERBB signaling AKT and ERK1/2 (Godinho et al., 2010). In addition, knockdown of the four ERBB receptors with specific siRNAs proved that BCAR4-induced cell proliferation is closely coupled to ERBB2 and ERBB3 signaling. The ERBB family of tyrosine kinase receptors have important roles in normal development, growth, differentiation, and tumorigenesis (reviewed in Holbro et al., 2003). Gene amplification and over-expression of ERBB2 has been reported in several types of cancer including breast, and has been shown to contribute to a poor clinical outcome. (Slamon et al., 1989; Gullick et al., 1991; Berns et al., 1992, 1995; Seshadri et al., 1993; Liu et al., 2007; Van Agthoven et al., 2009a). Overexpression or amplification of ERBB2 in primary breast cancers predicts response failure to tamoxifen therapy (Wright et al., 1992; Berns et al., 1995; Dowsett et al., 2001; Hurtado et al., 2008; Van Agthoven et al., 2009a). However, ERBB2 is also a target for established therapy (Hynes and Lane, 2005). The BCAR4 amino acid sequence has two predicted transmembrane domains, suggesting that it could be localized at cell membranes. So far, a direct ligand for ERBB2 has not been found (Baselga and Swain, 2009), and this might be due to the active conformation of the receptor (Garret et al., 2003).

BCAR4 might be a novel ligand for ERBB3, activating ERBB2/ ERBB3 heterodimers. How BCAR4 actually activates the ERBB2/ERBB3 signaling network remains to be established.

In conclusion, BCAR4 is a gene with oncogenic potential that transforms breast cancer cells into an estrogen-independent, antiestrogen-resistant phenotype. We showed that tamoxifen resistance induced by BCAR4 expression is independent of ESR1 function but compatible with ESR1 expression. Expression of BCAR4 was found in placenta and oocyte, possibly indicating an important function in these tissues. Due to its restricted pattern of expression and the fact that BCAR4 expression was also found in breast cancers (Meijer et al., 2006; Godinho et al., 2010), this novel gene may have clinical value in breast cancer therapy. Targeting BCAR4 would specifically affect the tumors expressing it and would probably cause little side effects because of its absence in normal adult tissues. The recently observed involvement of ERBB2/HER2 signaling in BCAR4-driven tamoxifen resistance (Godinho et al., 2010) indicates that specific targeting of the ERBB2 signaling pathway might provide an alternative treatment for patients having BCAR4-positive tumors.

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